Award Number: W81XWH-12-1-0376

TITLE: Protelytic Regulation of the Intestinal Epithelial Barrier: Mechanisms and Interventions

PRINCIPAL INVESTIGATOR: Toni M. Antalis

CONTRACTING ORGANIZATION: University of Maryland
Baltimore MD 21201-1531

REPORT DATE: September 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
SEPTEMBER 2015	Annual	1 Sept 2014 -31 Aug 2015

4. TITLE AND SUBTITLE
Protelytic Regulation of the Intestinal Epithelial Barrier: Mechanisms and Interventions

5a. CONTRACT NUMBER
W81XWH-12-1-0376

5b. GRANT NUMBER
PR110378

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Toni M. Antalis, Ph.D.

E-Mail: tantalis@som.umaryland.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Maryland Baltimore
Baltimore, Maryland 21201

8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Inflammatory bowel diseases are characterized by chronic, progressive and destructive inflammation of the gastrointestinal tract. The two main forms of inflammatory bowel diseases (IBD), Crohn's disease and Ulcerative Colitis, currently affect over 1 million Americans including military personnel, and the incidence among aging veterans is rising. Compromised intestinal barrier function underlies much of the pathology associated with many inflammatory bowel diseases. Matriptase is a membrane-anchored serine protease encoded by the *Suppression of Tumorigenicity-14* (*ST14*) gene that is required for epithelial barrier homeostasis. The project uses the *St14* hypomorphic mouse model of matriptase deficiency to 1) determine molecular mechanisms that mediate matriptase protection during DSS-induced experimental inflammatory colitis, 2) define molecular mechanisms by which matriptase becomes decreased during inflammatory colitis, and 3) investigate the importance of matriptase to cytokine induced barrier loss using an *in vitro* model of intestinal barrier repair. Our data demonstrate that the matriptase barrier forming pathway is down-regulated at the transcriptional and protein levels by cytokines produced during inflammatory colitis. Further, matriptase acts downstream of prostasin to mediate barrier formation and both of these proteases are coordinately regulated. The loss of this pathway is likely to facilitate intestinal barrier disruption in human IBD.

15. SUBJECT TERMS
Inflammatory bowel disease, colitis, intestinal barrier permeability, leaky gut

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	17	

TABLE OF CONTENTS:

INTRODUCTION:

Inflammatory bowel diseases (IBD) are characterized by chronic, progressive and destructive inflammation of the gastrointestinal tract. The two main forms of IBD, Crohn's disease and Ulcerative Colitis, currently affect over 1 million Americans including military personnel, and the incidence among aging veterans is rising. Compromised intestinal barrier function is believed to underlie much of the pathology of IBD. Matriptase is a membrane-anchored serine protease encoded by *Suppression of Tumorigenicity-14* (*ST14*) that is required for epithelial barrier homeostasis. Here, we are investigating matriptase dysregulation and its contribution to the pathogenesis of acute colitis using the *St14* hypomorphic mouse model of matriptase deficiency. Matriptase expression and regulation are being studied using the *St14* hypomorphic mouse and matriptase dysregulation is being investigated by subjecting *St14* hypomorphic and control mice to an experimental model of inflammatory colitis. The goal of the proposed studies is to determine the mechanisms by which matriptase protects gut barrier function in inflamed mucosa. The specific aims are (1) to determine the molecular mechanisms that mediate matriptase protection during experimental colitis by defining the effects on immune responses and epithelial cell regeneration and repair, (2) to define mechanisms responsible for down-regulation of matriptase in inflamed mucosa, and (3) to determine the contribution of matriptase loss to barrier dysfunction induced by cytokines associated with IBD pathogenesis.

KEYWORDS:

Intestinal barrier

Intestinal permeability

Crohn’s disease

Inflammation

Inflammatory bowel disease

Interleukin 4

Interleukin 13

Intestinal epithelial cells

Matriptase

Prostasin

Serine Protease

Type II transmembrane serine protease

Ulcerative colitis

OVERALL PROJECT SUMMARY:

We obtained approval for a one year extension without funds (EWOF) on August 24,2015. This will allow us to complete our planned research in year 4.

Research progress is aligned with respect to each task outlined in the SOW:

TASK 1: ***Seek regulatory approval for use of animals which includes the IACUC, University of Maryland, and the USAMRMC ACURO. Prior approval will also be obtained prior to any modifications of the protocol (months 1-36).***

The IACUC protocol was approved for this grant by the IACUC of the University of Maryland, Protocol #0913001 (expiration date 10/20/2016), and submitted to USAMRMC ACURO for approval by Sheron Westbrook of the USAMRMC Animal Care and Use Review Office prior to 01/27/2014. The most recent annual report was approved by the IACUC on 09/18/2015.

TASK 2: ***To determine the molecular mechanisms that mediate matriptase protection during mucosal inflammation in experimental colitis. (months 1-36).***

OBJECTIVE: To define mechanisms by which the intestinal epithelial barrier is protected by matriptase during inflammatory colitis. and the most recent annual report approved on 09/19/2014. The updated protocol

We hypothesize that the intrinsic defect in the intestinal barrier associated with the failure to form functional tight junctions in *ST14* hypomorphic mice (1; 2) prevents gut barrier repair and therefore perpetuates destructive intestinal inflammation associated with colitis and IBD, whereas normal matriptase levels in control mice during recovery enables essential barrier repair that promotes colitis resolution. Our experiments test this hypothesis utilizing a standard experimental mouse model of DSS-induced colitis. In this colitis model, the initiating trigger, DSS, causes injury to the epithelial layer provoking activation of an innate immune response to luminal contents and the induction of barrier repair processes. The experimental tasks are designed to investigate whether the increased permeability of ST14 hypomorphic intestinal epithelium causes recurring priming of the immune system with increased immune cell infiltration and activation, and/or whether diminished matriptase increases epithelial cell destruction and/or interferes with mechanisms of epithelial cell regeneration and repair.

The specific tasks are:
(a) To induce colitis in groups of ST14 hypomorph and control littermate mice by administration of DSS in drinking water, and then replace with water only. Mice will be monitored for body weights, survival and clinical symptoms (months 1-36).

Year 1 and 2 summary - This task requires experimental *St14* hypomorphic mice and control littermate mice obtained from our IACUC approved breeding protocol 0113002. During year 1, we encountered problems due to unusually low breeding likely caused by construction of a building near the animal facility. This problem was resolved towards the end of year 1 and initial studies were completed. Analysis of available mice treated with 2% DSS in drinking water for 7 days to induce colitis showed *St14* hypomorphic mice lost weight much more rapidly than their control littermates, and showed more severe symptoms of clinical disease. During year 2 we obtained sufficient numbers of mice to expand the time course of treatment of control and *St14* hypomorphic mice with 2% DSS to induce colitis. Due to increased potency of a new batch of DSS, we are treating mice with 2% DSS in drinking water for 5 days to induce comparable symptoms of colitis as observed previously at 7 days. We continued DSS treatment and tissue collection of control and *St14* hypomorphic mice at both Day 5 and Day 1.5 of treatment. *St14* hypomorphs were found to show increased clinical symptoms compared to control mice at both time points. Increased clinical symptoms at Day 1.5 in *St14* hypomorphic mice suggest that Matriptase expression can protect against the initiation of colitis. These data also confirmed our previous findings that matriptase deficiency in mice enhances disease severity.

Year 3 progress – We have continued to treat control and *St14* hypomorphic mice for 1.5 or 5 Days with 2% DSS and monitored body weights and clinical symptoms. Experiments have been performed with adequate numbers of control mice at both time points. This analysis showed that clinical symptoms do not become apparent until 5 days of DSS treatment (**Figure 1, top panel**). We have not completed treatment of *St14* hypomorphic mice at these time points due to their reduced viability which results in reduced availability of mice of this genotype per litter. These mice are now available and these studies will be completed in year 4.

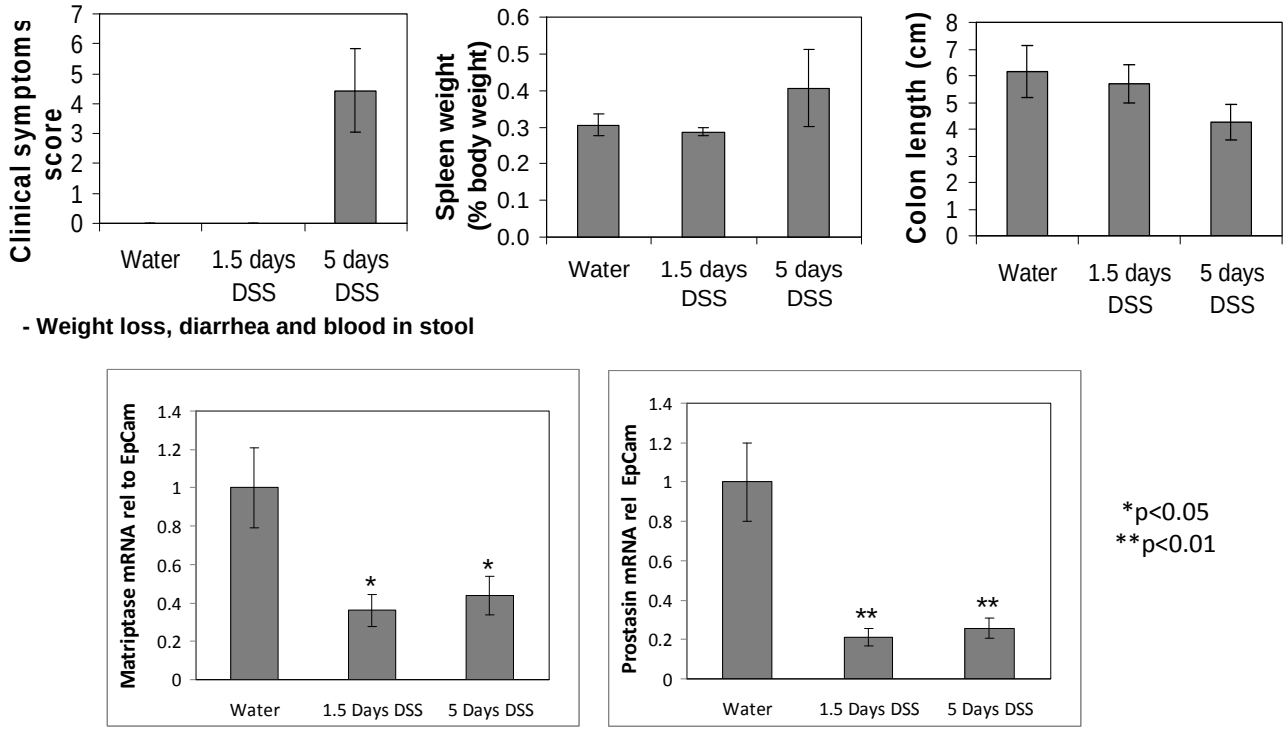


Figure 1. Matriptase and Prostasin mRNA are significantly down-regulated in colon tissues of control mice after 1.5 and 5 days of DSS induced colitis prior to the appearance of clinical symptoms. Symptoms do not appear until day 5 of DSS treatment. mRNA levels are detected by qPCR analysis and expressed relative to EPCAM mRNA after normalising both to GAPDH mRNA, n=6-8

(b) To sacrifice mice at various time points (3-10/group) during the course of the DSS protocol to evaluate molecular markers of acute inflammation in the subepithelial lamina propria, including quantity and nature of immune cell infiltration, and inflammatory cytokine production (months 1- 18)

Year 1 and 2 summary - Preliminary analysis of inflammation associated with the intestinal mucosa in *St14* hypomorphic mice was investigated by histological comparison of identical segments of colonic tissue after 2% DSS treatment for 1.5 and 5 Days. Analyses of H&E stained tissues of the distal colons for microscopic injury showed a) increased immune cell infiltrates in the mucosa and submucosa in both the *St14* hypomorphic and littermate control genotypes after DSS treatment, and b) increased loss of epithelium in the colons of *St14* hypomorphic mice compared with their corresponding control littermates, either due to increased epithelial sloughing/shedding or loss due to epithelial cell death. These possibilities will be further examined in Task 2(f). Preliminary analysis of cytokines and inflammatory mediators produced in response to DSS-induced colitis showed a) littermate control mice displayed an increase in TNFα after DSS treatment, which was not observed in *St14* hypomorphic mice, and b) IL-13 in *St14* hypomorphic mice increased relative to littermate controls, and this balance appeared to change after DSS treatment. Initial analyses also showed a slightly increased spleen size and shortened colon length, in both control and hypomorph mice after 5 days treatment, strongly indicative of an immune response.

Year 3 progress - We have continued to perform DSS treatments on increased numbers of control and hypomorph mice (1.5 days and 5 days) to collect tissues for H&E staining, immunohistochemistry for immune cell markers and Q-PCR analyses for cytokine expression. As stated above, increased numbers of

hypomorphic mice will be examined in year 4. Immunohistochemistry for immune cell markers and Q-PCR analyses for cytokine expression is on going, and will be completed in year 4.

(c) To sacrifice mice at various time points (3-10/group) during the course of the DSS protocol to assess gut permeability by serum FITC-dextran concentration after gavage and by ex vivo TEER measurements on colonic mucosa mounted transwell chambers. (months 1-18)

Year 1 and 2 summary - A 'leaky' mucosal barrier due to decreased barrier function is thought to underlie persistent and destructive mucosal inflammation in IBD and other intestinal disorders. In Year 1, we found that *St14* hypomorphic mice show enhanced baseline intestinal barrier permeability as measured by a) a 20% decreased baseline TEER of the distal colons of *St14* hypomorphic mice (1). In year 2 we expanded investigation of barrier permeability to examine intestinal permeability of St14 hypomorph mice compared with control littermates after DSS challenge 5 Days DSS challenge. The results showed that the defective epithelial barrier in *St14* hypomorphic mice contributes to enhanced permeability following DSS insult.

Year 3 progress - We have not yet performed permeability assays on Day 1.5 DSS treated mice. These assays will be performed in year 4.

(d) To sacrifice mice at various time points (3-10/group) during the course of the DSS protocol to evaluate bacterial translocation to lymph nodes, spleen and serum. (months 1-18)

From all mice that have been treated with DSS, we have collected tissue specimens from mesenteric lymph nodes, spleens and colons for analysis of bacterial translocation. In year 4 we will complete collection from the remaining experimental mice and measure bacterial translocation by qPCR amplification of bacterial DNA extracted.

(e) To sacrifice mice at various time points (3-10/group) during the course of the DSS protocol to assess differences in claudins and other junctional proteins that have been reported to form a "leaky" barrier in certain tissues between ST14 hypomorphic and control littermates. (months 1- 18)

Year 1 and 2 summary – In preliminary experiments from available mice, tissue segments from identical regions of the jejunum, small intestine, and the colons of littermate control and *St14* hypomorphic mice were collected for analysis of protein and mRNA expression. The initial analysis a) elevated claudin-2 protein expression in *St14* hypomorphs compared to littermate control mice, and b) no change in claudin-2 mRNA expression indicating that regulation of claudin-2 in the mouse colon may be largely post-transcriptional.

Year 3 progress - We continued to collect tissue specimens to continue these experiments. In year 4, once final mouse numbers are obtained we will complete the analysis.

(f) To sacrifice mice at various time points (3-10/group) during the course of the DSS protocol to compare markers of epithelial regeneration in the colonic mucosa of ST14 hypomorphic with control mice, including staining of distal colonic segments for Ki-67, BrdU label incorporation, and epithelial apoptosis (months 6-24)

Year 1 and 2 summary - Epithelial regeneration in the colonic mucosa of DSS-treated *St14* hypomorphic mice will be investigated by immunostaining of colonic segments for Ki-67, a nuclear protein preferentially expressed during active phases of the cell cycle and which is absent in resting cells (3), and secondly by injection of BrdU (50mg/kg) prior to sacrifice, which gets incorporated into dividing cells and can be immunostained as a marker of DNA synthesis (4; 5). Our preliminary analysis of colonic tissues from a limited number of untreated control and *St14* hypomorph mice showed strong positive staining for both Ki67 and BrdU by immunostaining, which appeared to be reduced in both genotypes after DSS treatment.

Year 3 progress - We continued to collect tissue specimens to perform this analysis on increased numbers of mice and at 0, 1.5 and 5 days DSS treatment. BrdU and Ki67 staining was successfully performed on all current colon specimens we have collected. Once tissues from remaining mice have been collected and immunostained, colonic crypts will be scored for BrdU-labeled cells and for Ki67 staining, and their position

recorded relative to the base of the crypt. We will investigate *in situ* cell death by TUNEL assay and activated caspase-3 staining of these tissue sections in year 4. In addition, we plan to expand more broadly to determine molecular mechanisms that are altered by matriptase deficiency through a more global analysis of signaling pathways.

TASK 3: ***To define mechanisms responsible for down regulation of matriptase in inflamed mucosa. (months 12-36).***

OBJECTIVE: To test the hypothesis that matriptase is down-regulated by cytokine-mediated inflammatory signaling pathways induced by immune system activation.

We and others have shown that loss of matriptase and protein expression disrupts barrier function (1; 2). Thus the mechanisms involved in the down-regulation of matriptase mRNA are of critical importance. The experimental tasks are designed to investigate how and when matriptase is regulated in inflamed mucosa and during barrier recovery using the *in vivo* β-gal reporter gene trap under the control of the matriptase promoter in both heterozygous and hypomorphic *St14* mice.

It was planned to focus on this task in Years 2 and 3 as proposed in the timeline.

The specific tasks are:

(a) To induce colitis in groups of 3-10 ST14 hypomorphic (-/GT) and control (+/GT) mice by administration of DSS for 5 days in drinking water and then replace with water only. Mice will be monitored for body weights and clinical symptoms (months 12-36)

Year 1 and 2 summary - In year 2 we obtained tissues from Day 5 DSS treated mice (2 control, 4 *St14* hypomorphs), Day 1.5 DSS treated mice (4 control, 4 *St14* hypomorphs), and untreated mice (3 control, 4 *St14* hypomorphs) that are suitable to detect β-galactosidase reporter activity.

Year 3 summary - We obtained tissues from sufficient numbers of tissues from untreated, Day 1.5 and Day 5 DSS treated control and *St14* hypomorphic mice to complete this study.

(b) To identify the time-dependent changes in matriptase promoter driven gene expression by X-gal staining, combined with co-staining approaches for molecular marker and cytokine expression. (months 12-18)

Year 1 and 2 summary - We found that tissues from untreated control mice (positive control tissues), that had been fixed with 4% PFA produced negative staining for β-galactosidase activity (Roche kit). Several different methods were employed to optimize detection of β-galactosidase activity on colonic tissues, and a protocol eventually successfully developed using a short (2 min) fixation in gluteraldehyde on cut frozen sections. Preliminary staining of 2 control and 2 St14 hypomorph mice, untreated or treated for 5 days with 2% DSS showed a reduction in X-gal staining in DSS treated mice compared to untreated mice of the same genotype. This data suggests matriptase expresssion is down-regulated at the level of transcription.

Year 3 progress - We collected further tissues from untreated, Day 1.5 and Day 5 treated mice, and have successfully stained tissues from untreated and Day 5 DSS treated control mice. We will complete the staining and analysis of St14 hypomorphic mice and Day 1.5 treated mice in year 4.

(c) To analyze tissue specimens by qPCR for total RNA levels and matriptase protein expression (months 12-24).

Year 1 and 2 summary - We initiated qPCR studies to investigate changes in matriptase mRNA expression in the absence and presence of colitis. Results from analysis of mice treated with 2% DSS for 5 days showed that matriptase expression is down-regulated in the colonic mucosa of control mice during DSS induced injury. We also found that the related protease prostasin which we found is important for matriptase activation in intestinal epithelium and essential for matriptase-mediated barrier formation (6), is also down-regulated by qPCR analysis. Both matriptase and prostasin mRNA show a statistically significant decrease during active colitis.

There was a possibility that the decreased levels of these proteases was a consequence of loss of epithelial cells during active colitis, and needed to be investigated by comparative analysis with EpCAM mRNA, an epithelial cell marker.

Year 3 progress - We completed qPCR analysis for EPCAM in colon tissues from control mice that had been treated with DSS for 5 days, and completed qPCR analysis on samples from 1.5 day DSS treatment for matriptase, prostasin and EPCAM. After normalizing mRNA levels of matriptase and prostasin to EPCAM, we found that both barrier promoting proteases are significantly down-regulated during both the initiation and duration of colitis (**Figure 1, lower panel)**. A significant finding is that the loss of protease expression is very rapid and precedes the appearance of clinical symptoms. This data showing a decrease in matriptase mRNA, is consistent with the observed loss of matriptase promotor-driven β-gal reporter expression. Together these data suggest that loss of this barrier forming pathway during colitis occurs at the level of reduced mRNA transcription.

Another goal of this task is to determine whether, in addition to mRNA down-regulation, matriptase protein expression is also lost over time in DSS-treated mice. In year 3 we attempted analysis of cell lysates made from frozen mouse colon sections by immunoblotting. Unfortunately, the 3 available commercial antibodies that detect matriptase by this method either did not work or detected non-specific bands on the western blots. We therefore tried another strategy using immunohistochemical staining approaches. We have now optimized detection of both matriptase and prostasin in paraffin embedded tissue sections of colons from untreated and DSS treated mice. The optimized protocol utilizes heat-induced antigen retrieval in citrate buffer pH 6, and immunostaining with primary and fluorescently labeled secondary antibodies. Sections are co-stained with the nuclear marker DAPI. We find Matriptase is found to concentrate at epithelial junctions of the colonic villi **(Figure 2)**, while prostasin has a more diffuse and apical location in villus epithelial cells **(Figure 2)**, both consistent with their localization in human intestinal epithelium. Preliminary results also suggest protein expression of both proteases are lost during active colitis. All sections are currently being stained and fluorescent signals will be quantitated using ImageJ analysis, and levels normalized for cell number using the DAPI signal.

Since we observed that the matriptase-activating protease prostasin (6) is also lost during active colitis in this model, we sought to determine whether prostasin, like matriptase, is also lost during human colitis. This was assessed using an mRNA array of human colon tissue samples from normal, ulcerative colitis and Crohns' disease patients (Origene). We found that both proteases are also down-regulated in human colitis (**Figure 3**), making our findings in the murine model likely to be physiologically relevant to human disease, and emphasizing the importance of studying this pathway in maintenance of intestinal barrier function. Given the low number of normal tissues in these arrays we hope to analyze further samples in Year 4.

(d) To correlate matriptase gene expression with cytokine profiles to identify effectors of matriptase down-regulation during inflammation. (months 12-24)

The RNA samples that have been prepared for qPCR anaysis of matriptase and prostasin will be used in qPCR analysis for cytokines known to be implicated in human colitis will be performed in year 4.

(e) To treat mice with antibiotics for a 4 week period prior to DSS challenge. (months 18-36)

This experiment is in progress and will be completed in year 4.

(f) To establish whole colon cultures from - /GT mice and +/GT mice and assess viability and efficacy for ex vivo studies. (months 12-36)

These experiments have been initiated. We have encountered technical problems with explant viability that we need to overcome.

(g) To investigate specific regulation of the matriptase gene activity by specific inflammatory cytokines and incorporating cytokine pathway inhibitors (months 12-36)

Data obtained using animal studies (Task 3(d)) and from cell culture systems (Task 4) will be used to guide the selection of inflammatory mediators and their inhibitors to be tested in these experiments in year 4.

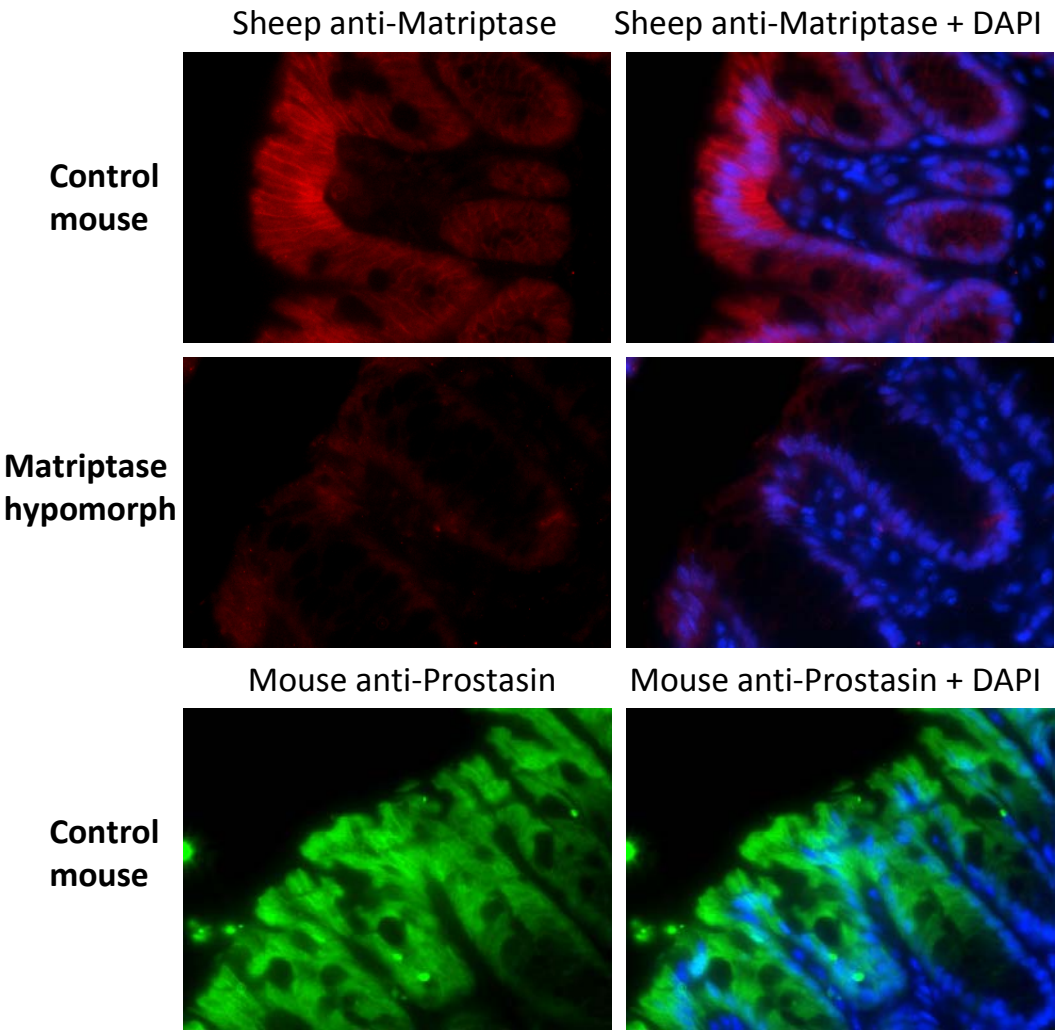
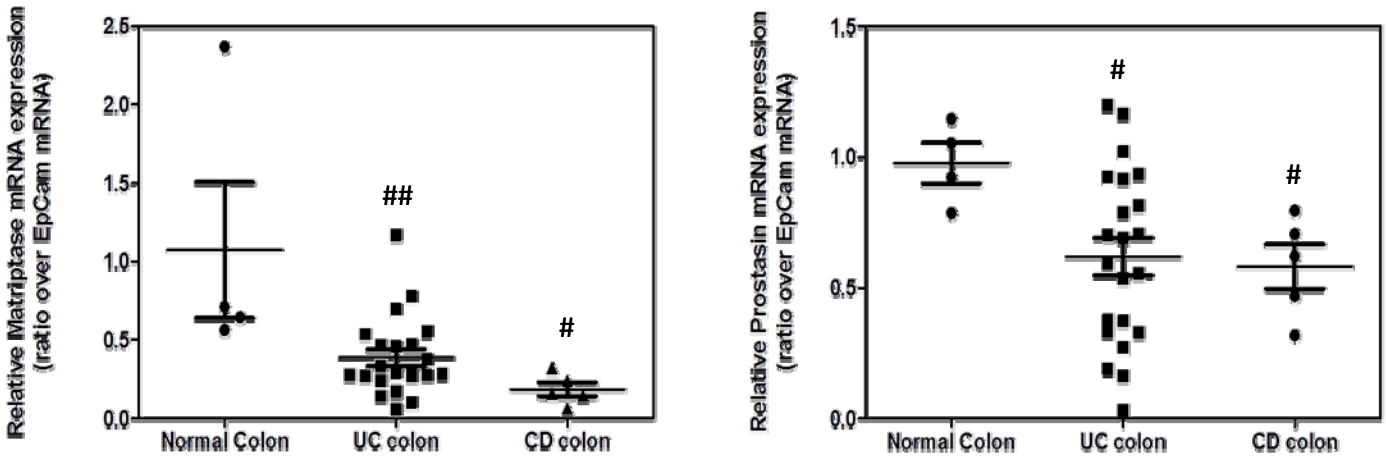


Figure 2. Detection of Matriptase and prostasin protein in mouse colon tissue sections. Colon sections from control or matriptase hypomorph mice were immunostained with sheep anti-matriptase antibody followed by anti-sheep Alexa 549, or mouse anti-prostasin followed by anti-mouse Alexa 488. Matriptase hypomorph mice express much lower matriptase protein than control mice as expected



#$p \leq 0.05$, ##$p < 0.01$

Figure 3. Matriptase and prostasin are significantly down-regulated in human colitis. qPCR analysis of matriptase and prostasin in colonic tissue from normal, ulcerative colitis (UC) and Crohns' disease (CD) patients. Protease expression was normalized to the epithelial marker EPCAM.

TASK 4: ***To test the hypothesis that matriptase loss, induced by IL-13 during inflammation, mediates the IL-13 mediated effects on increased permeability and delayed epithelial barrier repair via derangement of tight junction integrity, increasing expression or localization of claudin-2 or other TJ proteins and stimulation of epithelial cell apoptosis (months 1-18).***

OBJECTIVE: To test the hypothesis that matriptase loss, induced by IL-13 during inflammation, mediates the observed IL-13 effects on increased permeability and delayed epithelial barrier repair.

This task aims to identify a possible mechanism by which the IL-13/STAT6 signaling pathway induces apoptosis and barrier disruption. IL-13 production and claudin-2 expression are both increased in human ulcerative colitis and Crohn's disease (7; 8), and matriptase is decreased (1). We will investigate the hypothesis that IL-13 induced down-regulation of matriptase contributes in a significant way to IL-13 dependent barrier dysfunction.

The specific tasks are:
(a) To grow cultures of polarized Caco-2 and T84 cells on permeable transwell supports and treat with IL-13 for various times (months 1-3).

After initial difficulties with variability in Caco-2 cultures, we have successfully been able to grow polarized Caco-2 and T84 cells on transwell supports and treat with IL-13 for various times.

(b) To monitor the integrity of the barrier by measurement of TEER and by the flux of 4kDa FITC conjugated dextran across monolayers (months 1-3)

Year 1 and 2 summary - Preliminary data showed that after treatment of polarized T84 monolayers with IL-13, barrier disruption occurred as monitored by a reduction in TEER and an increase in monolayer permeability to macromolecular FITC-dextran. We have been able to consistently induce barrier disruption of T84 cultures using IL-13 in combination with the related Th2 type cytokine IL-4, which is also up-regulated in active colitis.

Year 3 progress - We have also investigated T84 barrier disruption by the Th1 cytokines IFNg and TNF which are also increased in human colitis. Barrier disruption is induced by both treatments shown by loss of TEER (**Figure 4A**) and increased monolayer permeability to FITC-dextran (**Figure 4B**). We have been able to induce barrier disruption of Caco-2 monolayers with IL-4/IL-13 (**Figure 5**).

(c) To investigate the effects of the restoration of recombinant matriptase to cell monolayers and determine the effects on IL-13 induced barrier permeability, claudin-2 levels, apoptosis, STAT6 phosphorylation and restitution rate in vitro (months 3-12)

Year 1 and 2 summary - We established T84 culture models showing loss of both matriptase and prostasin protein occurs during IL-4/IL-13 induced barrier disruption (**Figure 4C**). Analysis of cultures over time showed that the mRNA for matriptase is down regulated within 6 hours and remains low during the course of cytokine treatment. Similarly, prostasin mRNA is also rapidly down regulated by cytokine treatment but is almost recovered by 24 hrs. These data are consistent with the reduced matriptase transcription observed after DSS-induced colitis *in vivo.* We also found that claudin-2 is up-regulated by IL-4/IL-13 treatment (**Figure 4C**), consistent with the up-regulation that occurs during matriptase deficiency. Stat-6 was activated by IL-4/IL-13 treatment as expected. We determined that IL-4/IL-13 did not induce cell death indicated by PARP cleavage (**Figure 4C**).

Year 3 progress –. We have compared the effect of IL-4/IL-13 to that of TNF/IFNγ on barrier disruption and protease expression. These cytokines also induced the loss of matriptase and prostasin expression (**Figure 4C**). In contrast to IL-4/IL-13, TNF/IFNγ did not induce the up-regulation of claudin-2, and also induced cell death (**Figure 4C**). This induction of cell death complicates the study of effects on barrier function and protease loss, and therefore have not pursued this further. We have also solidified our time course analysis of protease loss during IL-4/IL-3 treatment by qPCR and protein analysis in replicate experiments. These findings are currently being confirmed using the Caco-2 cell model. Experiments to investigate the effects of restoration

of recombinant matriptase on the IL-13 mediated barrier disruption are ongoing. We have encountered some difficulty with these experiments since we observed that the recombinant matriptase is inhibited by factors in serum. While Caco-2 cells are able to maintain barrier function in serum free media, we found that T84 cells lost TEER once they were placed in serum free media (OPTIMEM). We have now found a serum free media condition in which T84 cells can maintain barrier, and these experiments are in progress.

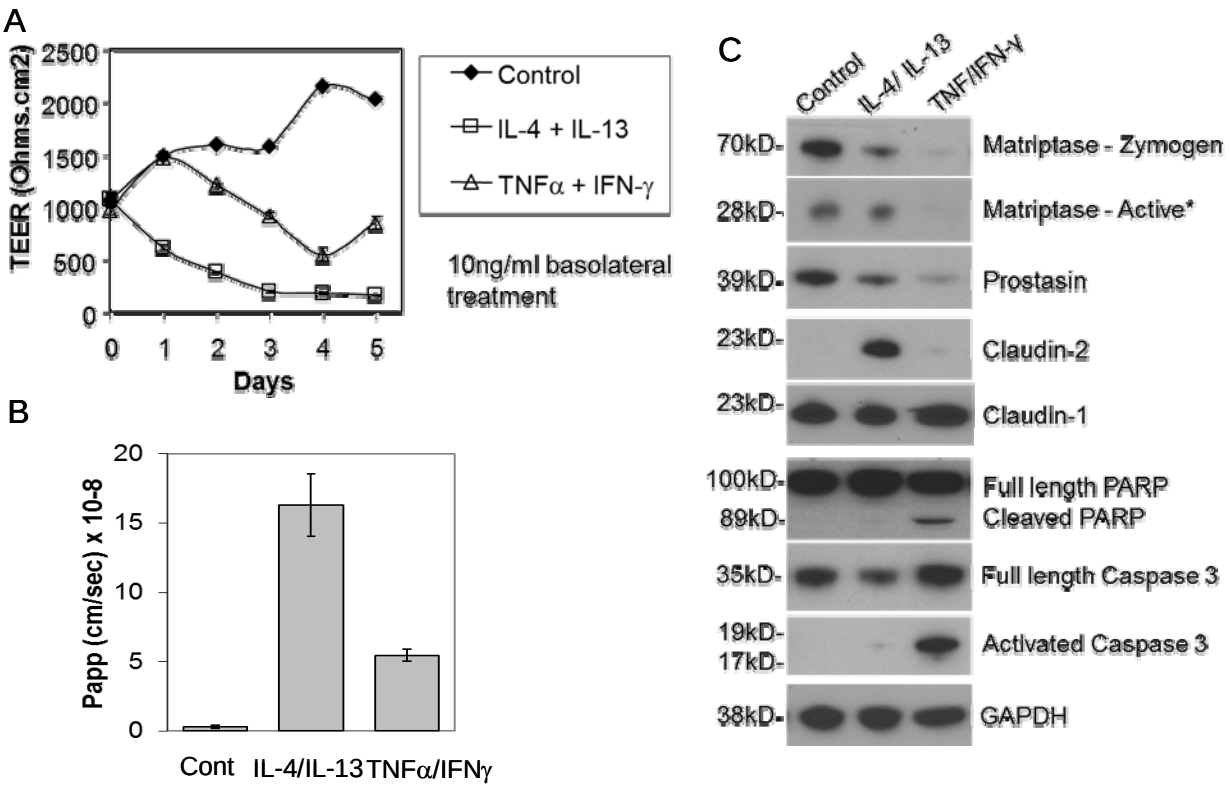


Figure 4. Cytokines induce barrier disruption of polarized T84 monolayers. T84 cells were allowed to develop barrier function over 7 days, then treated with the indicated cytokines once TEER had reached 1000 Ohms.cm^2. Both treatments induced barrier disruption demonstrated by loss of TEER (A) and increased permeability to 4 kDa FITC dextran. (C) Immunoblot analysis for the indicated proteins from cell lysates obtained after 5 days of cytokine treatment. Graphs show mean +/- SEM from triplicate wells.

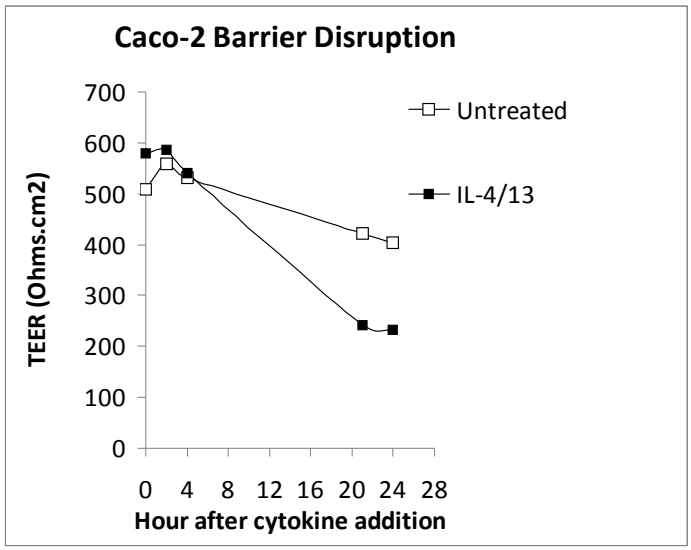


Figure 5. IL-4//IL-3 induce barrier disruption in Caco-2 monolayers. Barrier disruption induced by IL-4/IL-13 assessed by TEER.

(d) To transfect epithelial monolayers with pcDNA plasmid vectors encoding human matriptase and control constructs, generate and characterize stable cell lines, and determine the impact on IL-13 induced barrier permeability, claudin-2 levels, apoptosis, STAT6 phosphorylation and restitution rate in vitro (months 4-18)

Year 1 and 2 Summary – Preliminary studies in which SW480 cells (which do not express endogenous matriptase) were transfected with human matriptase cDNA in pcDNA3.1 and vector alone, showed enhanced transmigration across wounded monolayers, suggesting re-expression of exogenous matriptase by transfection is able to mediate matriptase functions. Studies were planned to investigate T84 restitution in the absence and presence of IL-13 in the presence or absence of exogenously expressed matriptase that is not under the control of it's normal promotor, which is sensitive to down-regulation induce by these cytokines. To achieve this, we designed an expression strategy to allow detection of cells expressing the transfected (ectopic) matriptase, distinct from the endogenous matriptase.

Year 3 progress - cDNAs of matriptase and prostasin (and their inactive mutants) were cloned into pIRES-EF1a-GFP or pIRES-EF1a-dsRed expression vectors. These vectors express a bi-cistronic mRNA so that the protease and a fluorescent molecule are produced from the same transcript and allows detection of cells expressing these proteases exogenously. Expression of the vectors was confirmed in transient transfections in HEK293 cells. Transient transfections using Lipofectamine were performed into T84 and Caco-2 cells and a fluorescent signal could be detected in ~20% of cells. Cells were then selected with G418 for 10 days. Unfortunately after 2 further passages, image and FACS analysis showed that after selection <1% of cells had retained expression of the vectors. We have since optimized transfection using the NEON transfection system (Invitrogen) and have been able to find conditions where 85-95% of cells are able to be transiently transfected. **Figure 6** shows an example of GFP transfection into both T84 and Caco-2 cells 48 hours post-transfection. Given this high level of expression, we plan to use these cultures to plate directly onto transwell filters to complete the experiment, rather than creating stable lines. These cultures will then be used to assess whether constitutive expression of matriptase can protect against IL-13-induced barrier disruption.

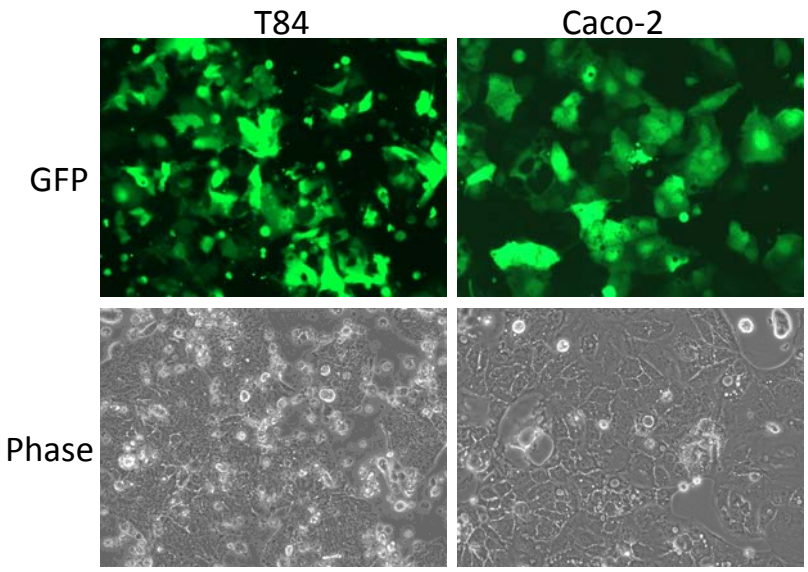


Figure 6. Colonic cell lines tranfected using the NEON transfection system expressing GFP. Electroporation conditions were optimized. Images are from 48h post-transfection.

TASK 5: ***Perform data analysis and prepare technical reports (months 1-36).***

We are continuing data analysis, writing up the results and planning publication of the findings.

KEY RESEARCH ACCOMPLISHMENTS:

- Matriptase plays a critical role in epithelial barrier formation and assembly.
- Matriptase deficiency leads to persistent, severe inflammatory DSS-induced colitis.
- Persistent colitis in ST14 hypomorphic mice is associated with an inability to recover TEER.
- Matriptase and it's activator prostasin are down-regulated in intestinal mucosa during experimental DSS-induced colitis, and in human colitis.
- Matriptase and prostasin are down-regulated by IL-13 implicated in the pathogenesis of IBD
- Loss of the prostasin-matriptase barrier forming pathway during colitis occurs at the level of reduced mRNA transcription in both an animal model, in experimental intestinal epithelial monolayers and in human disease.

CONCLUSION:

Compromised intestinal barrier function is strongly associated with the pathogenesis of IBD. This project seeks to understand a critical new mechanism by which intestinal epithelial barrier function can be compromised during inflammatory colitis that occurs during IBD. The enhanced susceptibility of matriptase hypomorph mice to DSS-induced injury and inflammatory colitis combined with the *in vitro* assays of barrier function, suggest that matriptase could be an important contributor to inflammatory colitis and repair of injured intestinal mucosa. Our data to date show that matriptase deficiency in mice enhances disease severity and suggest that the nature of inflammation may be altered by the presence and absence of matriptase. These data support the notion that the defective epithelial barrier in *St14* hypomorphic mice likely leads to persistent inflammation following DSS insult. During this third year of the grant, we have continued to obtain sufficient tissue specimens for detailed molecular characterization of changes induced by DSS colitis and repair processes in St14 hypomorphic and control mice (Task 2), and have completed the *in vivo* matriptase and prostasin mRNA regulation analysis. A significant finding is that loss of these proteases precedes the appearance of clinical symptoms in murine colitis, suggesting their loss may significantly contribute to disease susceptibility. We also determined that both proteases are also down-regulated in human colitis (Task 3). We have continued the studies on the mechanisms involved in IL13 regulation of experimental epithelial barriers and permeability with a focus on the role of matriptase (Task 4).

PUBLICATIONS, ABSTRACTS AND PRESENTATIONS :

a) Manuscripts:

Netzel-Arnett S, Buzza MS, Shea-Donohue T, Désilets A, Leduc R, Fasano A, Bugge TH, Antalis TM. Matriptase protects against experimental colitis and promotes intestinal barrier recovery. Inflamm Bowel Dis. 2012 Jul;18(7):1303-14. PMCID: PMC3288858.

Buzza MS, Martin EW, Driesbaugh KH, Désilets A, Leduc R, Antalis TM. Prostasin is required for matriptase activation in intestinal epithelial cells to regulate closure of the paracellular pathway. J Biol Chem. 2013 Apr 12;288(15):10328-37. PMCID: PMC3624416

b) Abstracts:

Buzza MS, Johnson T, Conway GD, Martin EW, Shea-Donohue T and Antalis TM. The Role of the Prostasin-Matriptase Membrane-Anchored Serine Protease Cascade in Protection of Intestinal Epithelial Barrier Function during Inflammation. FASEB SRC on Proteases in Hemostasis & Vascular Biology, Keystone, CO, 2015.

Buzza, MS, Conway, GD, Martin, EW, Shea-Donohue, T, and Antalis, TM. 'Proteolytic Regulation of the Intestinal Epithelial Barrier: Mechanisms and Interventions' 2014 Military Health system Research symposium (MHSRS).

Johnson T, Buzza M, Conway GD, Antalis TM 'Loss of the barrier forming membrane-anchored serine protease Matriptase during gastrointestinal inflammation- potential role in disease pathogenesis. Fifth Annual Cancer Biology Research Retreat, 2014, University of Maryland Baltimore.

Buzza, M.S., Conway, G.D., Martin, E.W., Shea-Donohue, T. and Antalis, T.M. 'Inflammation-induced loss of the Prostasin-Matriptase barrier promoting pathway in intestinal epithelium contributes to the pathogenesis of colitis'. Gordon Research Conference on Plasminogen Activation and Extracellular Proteolysis, Feb 2014, Ventura, CA

Buzza, M.S., Martin, E.W., Driesbaugh, K., Desilets, A., Leduc, R., and Antalis T.M. 'Prostasin is required for Matriptase activation during the formation and maintenance of the intestinal epithelial barrier'. ASBMB Special Symposia Series, Membrane-Anchored Serine Proteases, Sept 2013, Potomac, MD.

c) Presentations made:

1. Buzza, Marguerite, 'The Role of the Prostasin-Matriptase Membrane-Anchored Serine Protease Cascade in Protection of Intestinal Epithelial Barrier Function during Inflammation. FASEB SRC on Proteases in Hemostasis & Vascular Biology, Keystone, CO, June 7-12, 2015 (Poster)

2. Antalis, Toni, 'Proteolytic Regulation of the Intestinal Epithelial Barrier: Mechanisms and Interventions' 2014 Military Health system Research symposium (MHSRS), Harbor Beach Marriott, Fort Lauderdale, Florida, August 18-21, 2014 (Poster)

3. Johnson, Tierra, 'Loss of the barrier forming membrane-anchored serine protease Matriptase during gastrointestinal inflammation- potential role in disease pathogenesis. Fifth Annual Cancer Biology Research Retreat, UMB SMC Campus Center, University of Maryland Baltimore, June 9, 2014 (Poster)

4. Buzza, Marguerite, 'Inflammation-induced loss of the Prostasin-Matriptase barrier promoting pathway in intestinal epithelium contributes to the pathogenesis of colitis'. Gordon Research Conference on Plasminogen Activation and Extracellular Proteolysis, Feb 2014, Ventura, CA (Oral presentation)

5. Antalis, Toni, 'Membrane-Anchored Serine Proteases in Inflammation and Protease Activator Receptor Signaling' Gordon Conference on Plasminogen Activation and Extracellular Proteolysis, Ventura, CA, February 2014 (Oral presentation)

6. Buzza, Marguerite, 'Prostasin is required for Matriptase activation during the formation and maintenance of the intestinal epithelial barrier'. ASBMB Special Symposia Series, Membrane-Anchored Serine Proteases, Sept 2013, Potomac, MD (Oral presentation)

7. Antalis, Toni, 'Prostasin is required for matriptase activation in intestinal epithelial cells to regulate closure of the intestinal epithelial barrier" XIVth International Workshop on the Molecular and Cellular Biology of Plasminogen Activation, June 4-8, 2013**,** Notre Dame, Indiana (Oral presentation)

INVENTIONS, PATENTS AND LICENSES : nothing to report

REPORTABLE OUTCOMES: nothing to report

REFERENCES:

1. Buzza, M. S., Netzel-Arnett, S., Shea-Donohue, T., Zhao, A., Lin, C. Y., List, K., Szabo, R., Fasano, A., Bugge, T. H., and Antalis, T. M., "Membrane-anchored serine protease matriptase regulates epithelial

barrier formation and permeability in the intestine," *Proc.Natl.Acad.Sci.U.S.A*, Vol. 107, No. 9, 2010, pp. 4200-4205.

2. List, K., Kosa, P., Szabo, R., Bey, A. L., Wang, C. B., Molinolo, A., and Bugge, T. H., "Epithelial integrity is maintained by a matriptase-dependent proteolytic pathway," *Am.J.Pathol.*, Vol. 175, No. 4, 2009, pp. 1453-1463.

3. Scholzen, T. and Gerdes, J., "The Ki-67 protein: from the known and the unknown," *J.Cell Physiol*, Vol. 182, No. 3, 2000, pp. 311-322.

4. Houle, C. D., Peddada, S. D., McAllister, K. A., Ward, T., Malphurs, J., Gersch, W. D., and Davis, B. J., "Mutant Brca2/p53 mice exhibit altered radiation responses in the developing mammary gland," *Exp.Toxicol.Pathol.*, Vol. 57, No. 2, 2005, pp. 105-115.

5. Fukata, M., Michelsen, K. S., Eri, R., Thomas, L. S., Hu, B., Lukasek, K., Nast, C. C., Lechago, J., Xu, R., Naiki, Y., Soliman, A., Arditi, M., and Abreu, M. T., "Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis," *Am.J.Physiol Gastrointest.Liver Physiol*, Vol. 288, No. 5, 2005, pp. G1055-G1065.

6. Buzza, M. S., Martin, E. W., Driesbaugh, K. H., Desilets, A., Leduc, R., and Antalis, T. M., "Prostasin is required for matriptase activation in intestinal epithelial cells to regulate closure of the paracellular pathway," *J.Biol.Chem.*, 2013.

7. Heller, F., Florian, P., Bojarski, C., Richter, J., Christ, M., Hillenbrand, B., Mankertz, J., Gitter, A. H., Burgel, N., Fromm, M., Zeitz, M., Fuss, I., Strober, W., and Schulzke, J. D., "Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution," *Gastroenterology*, Vol. 129, No. 2, 2005, pp. 550-564.

8. Prasad, S., Mingrino, R., Kaukinen, K., Hayes, K. L., Powell, R. M., MacDonald, T. T., and Collins, J. E., "Inflammatory processes have differential effects on claudins 2, 3 and 4 in colonic epithelial cells," *Lab Invest*, Vol. 85, No. 9, 2005, pp. 1139-1162.

9. Rosen, M. J., Frey, M. R., Washington, M. K., Chaturvedi, R., Kuhnhein, L. A., Matta, P., Revetta, F. L., Wilson, K. T., and Polk, D. B., "STAT6 activation in ulcerative colitis: A new target for prevention of IL-13-induced colon epithelial cell dysfunction," *Inflamm.Bowel.Dis.*, 2011.